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Antiviral Activities of New Cidofovir Analogs Against Camelpox Virus, Used as a Model of Variola Virus, in Human Skin Equivalent Cultures

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Variola virus (VARV) is a member of the *Orthopoxvirus* genus that also includes camelpox virus (CMLV). Growing concerns over the possible release of VARV as a biological weapon have stimulated the development of new antiviral therapies to treat post-exposure VARV infection. CMLV is the etiologic agent of an orthopoxvirus infection in camels and dromedaries and has been shown to be the closest known orthopoxvirus to VARV. In this study, we have evaluated the potency of the three classes of acyclic nucleoside phosphonates (ANPs) against CMLV in human embryonic lung (HEL) cells and primary human keratinocyte (PHK) monolayers, as well as in a three-dimensional skin equivalent model for epitheliotropic viruses. The concentrations required to inhibit 50% of viral replication (IC_{50}) were determined by plaque reduction assays. We found seven active compounds from the 1st class of ANPs including cidofovir (Vistide®) and from the 2nd class of ANPs that includes the DAPy derivatives with IC_{50} values ranging from 0.04 μg/ml to 2.78 μg/ml in both HEL and PHK cells. The 3rd class of ANPs containing a 5-azacytosine moiety were active against CMLV in HEL cells with IC_{50} values ranging from 0.04 μg/ml to 6.40 μg/ml, giving selectivity indices from 20 to 130. The antiviral activities of the relevant molecules were confirmed in virus yield assays in monolayers. We finally used human organotypic epithelial rafts cultures to evaluate the antiviral activities of the compounds. This model gives histological pictures comparable to those described for the skin biopsy specimens of the corresponding diseases. Complete suppression of cytoplasmic ballooning of the keratinocytes caused by CMLV was observed at the highest concentrations of the ANPs tested so far. In conclusion, we have established an in vitro camelpox virus infection model for the evaluation of anti-poxvirus compounds.

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Cytopathic Maporal Hantavirus Infection of Vero E6 Cells

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Hantavirus cardiopulmonary syndrome (HCPS) is an acute human disease with remarkably high case fatality rates (30–50%). Maporal virus (MAPV), recently isolated from western Venezuela, is most similar phylogenetically to hantaviruses known to cause HCPS in southern regions of South America. There is no evidence that MAPV can productively infect humans and cause severe disease, yet infection in hamsters closely resembles disease manifestations associated with human cases of HCPS. In general, hantaviruses, produce little, if any, cytopathic effect (CPE) in cultured cells. Unexpectedly, we found that MAPV produces dramatic CPE in Vero E6 cells resulting in rapid and complete monolayer destruction. Cell death was triggered through the induction of apoptosis as demonstrated by caspase 3/7 activation and TUNEL staining following infection. Blockade of apoptosis by the caspase inhibitor Z-VAD-FMK during MAPV infection limited cytopathology and cell death, with little effect on viral burden. Induction of apoptosis may require active viral replication as inhibitory effects of ribavirin and consensus a-interferon protected cells challenged with MAPV. As with other pathogenic hantaviruses, MAPV was found to utilize the αvβ3 integrin for cellular entry suggesting that it may also be a human pathogen. Since infectivity could not be entirely blocked with specific antibodies, other receptors may be involved. Highly cytopathic MAPV infection in Vero E6 cells will greatly facilitate the development of high-throughput cell-based screening assays needed to identify effective antivirals for the treatment of severe hantavirus infections.

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Comparison of Anti-Proliferative Activity of Selected Antiviral Agents in Various Assay Systems

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Determining toxicity of experimental compounds is an important step in the antiviral screening process. While bone marrow clonogenic assays are considered the most predictive for drug induced bone marrow suppression, they are expensive and not

readily available for routine drug screening. Our laboratory has utilized a cell proliferation assay in Human Foreskin Fibroblast (HFF) cells that is predictive for toxicity in bone marrow. This labor intensive method uses a Coulter counter to enumerate live cells and is the current method of choice in our laboratory. We have compared this assay with three other assay systems in an attempt to automate the assay. Cellular replication was measured in HFF cells using a neutral red uptake assay, a crystal violet/formalin stained assay, a luminescent assay (CellTiter-Glo, Promega), and our standard cell counting assay. These experiments were conducted in both HFF and human embryonic lung (HEL-299) cells to see if results were cell line dependent. The neutral red uptake assay is based upon the uptake of a vital dye by living cells, whereas crystal violet stains all adherent cells. CellTiter-Glo generates a signal proportional to the amount of ATP present, which is proportional to the number of cells present. We compared a panel of drugs with well-characterized toxicities in each of the assay systems. Comparison of the IC_{50} values from the various assays demonstrated that the cell counting method using a Coulter counter was the most sensitive and predictive of toxicity in bone marrow. The neutral red uptake and the luminescent assay were less sensitive, while the crystal violet staining was relatively insensitive. There was no significant difference in the HFF and HEL-299 cells regardless of which method was utilized.

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Synthesis and Structure Activity Relationships among Non-nucleoside Analogs of Toyocamycin Active against Herpesviruses

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Toyocamycin (4-amino-5-cyano-7-β-D-ribofuranosylpyrrolo [2,3-*d*]pyrimidine) is a cytotoxic nucleoside. In contrast, certain of its deoxyribosyl, arabinosyl and acyclic analogs are less or non-cytotoxic. Some of these analogs have potent activity against HSV and HCMV (Renau et al., 1996. J. Med. Chem. 39, 873). The compounds act by a unique mechanism (Jacobson et al., 1999. Antimicrob. Agnts. Chemother. 43, 1888) early in the viral replication cycle (Evers et al., 2004. Antimicrob. Agnts. Chemother. 48, 3918). The simplest of these, the 7-methyl toyocamycin analog, was neither active against herpesviruses nor cytotoxic. The compounds that had antiviral activity, however, also had some cytotoxicity. Consequently we extended the original research and now report the synthesis and antiviral activity of other 7-alkyl analogs that also are substituted in the 6-position. These compounds were synthesized in a manner similar to that described by Renau et al. Using this and an analogous procedure, a series of 4-amino-5-cyano-pyrrolo[2,3-*d*]pyrimidines was synthesized with either H, Br, or NH2 in the 6-position and alkyl groups from methyl to octyl in the 7-position. These changes affected activity against HSV-1, HCMV, and cytotoxicity. In contrast to the 6-unsubstituted- and 6-Br-7-methyl analogs that were inactive, the 6-NH2 compound was active against both HSV-1 and HCMV (IC50's = 25 and 2 μM, respectively). Increasing the length of the 7-alkyl group increased activity against both viruses with propyl or butyl being optimal. Although the 6-Br-7-methyl analog was inactive at 100 μM against both viruses, 6-Br-7-ethyl and longer 7-alkyl analogs were active in the low to sub-micromolar range. The 6-NH2 analogs exhibited some cytotoxicity at 20–70 μM whereas the 6-Br analogs were not cytotoxic at 100 μM. Consequently, we conclude that compounds such as the 6-Br-7-butyl analog have specific antiviral activity against HSV-1 and HCMV.

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Combinations of CMX-001 and ST-246 Synergistically Inhibit Orthopoxvirus Replication In Vitro

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The necessity for the development of compounds for use in the treatment of orthopoxvirus infections originating from either a bioterror release or a natural endemic infection has yielded several drug candidates. A few of these are under active development for the treatment of orthopoxvirus infections including ST-246 and CMX-001 (HDP cidofovir), that are highly active both in vitro and in vivo. Our experiments were designed to determine if combinations of these two drugs would result in enhanced efficacy since: (1) they are the most advanced candidates under development, (2) they have different mechanisms of action and might be expected to act synergistically, and (3) they potentially could be used together in the clinic to avoid certain issues such as drug resistance. Combination assays were initially performed in human foreskin fibroblast (HFF) cells using the Copenhagen strain of vaccinia virus. Results from these studies revealed a robust synergistic interaction against viral replication suggesting that this drug combination might be particularly effective. Simultaneous cytotoxicity controls did not reveal any increased toxicity and suggested that it was a true antiviral effect. Treating viral infections with combinations of drugs with different mechanisms of action is advantageous because the combinations can offer improved efficacy at lower